

## Research article

**Nephrin a biomarker of early glomerular injury in newly diagnosed untreated hypertensive subjects**Anitha Devanath<sup>1</sup>, Jayakumari S.<sup>1</sup>, Seena Sankar<sup>2</sup>, Shubha N. Prakash<sup>1</sup><sup>1</sup>Department of Biochemistry, <sup>2</sup>Department of General Medicine, St. John's Medical College, Bengaluru, 560034, Karnataka, India

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Corresponding author: **Anitha Devanath**. Email: anitha.d@stjohns.in**ABSTRACT**

**Introduction and Aim:** Hypertension and proteinuria is known to cause renal and cardiovascular disease and mortality in patients irrespective of diabetes. It is beneficial to identify proteinuria and probable glomerular injury early to take preventive measures from cardiovascular event. In our study, we aimed to evaluate whether a biomarker such as nephrin can detect early glomerular injury in treatment naïve hypertensive subjects.

**Materials and Methods:** Forty newly diagnosed, treatment naïve hypertensive subjects were recruited for the study along with 40 normotensive controls after obtaining informed consent and procuring approval from Institutional Ethics Committee. The hypertensive group was classified as diabetic and non-diabetic hypertensives and compared with apparently healthy controls (normotensive). Urine sample was analyzed for microalbumin, creatinine and nephrin. Blood sample was analyzed for glycated hemoglobin, urea, creatinine, sodium, and potassium. Statistical analysis was performed using ANOVA to compare the groups for various parameters. Odds ratio was calculated.

**Results:** Hypertensives were sub-grouped based on amount of microalbumin excreted. Urine nephrin excretion was significantly higher in hypertensive subjects than normotensive subjects (nephrin cut-off: 0.09 mg/g of creatinine). Urine nephrin (mg/g) was found to be elevated (median 0.15; interquartile range, 0.12 and 0.17) in hypertensives with normoalbuminuria and it was significantly higher than normotensive subjects (median 0.07; interquartile range, 0.04 and 0.09).

**Conclusion:** Urine nephrin may be used as a biomarker of early glomerular injury in hypertensive subjects even before microalbuminuria is detected.

**Keywords:** Nephrin; treatment naïve hypertensive; ACR; microalbuminuria.

**INTRODUCTION**

Hypertension is known to be associated with proteinuria (>150 mg/day urine protein excretion). Prevalence of microalbuminuria (excretion of urine albumin > 30 mg/day) in patients with hypertension varies from 8 to 23% (1). Studies have shown that hypertension irrespective of diabetic or non-diabetic status is indicative of cardiovascular and renal disease that eventually develop into End Stage Renal Disease (ESRD) (2,3). General population studies such as Aus-Diab and Prevention of Renal and Vascular End Stage Disease (PREVEND) has showed an 8 to 11.5% prevalence of microalbuminuria in individuals with hypertension (4). The Losartan Intervention for Endpoint Reduction (LIFE) trial in hypertensive patients with electrocardiographic signs of left ventricular hypertrophy showed a 23% prevalence (1). Recent data from PREVEND (4) showed an 8% incidence of an individual moving from a normoalbuminuria to a microalbuminuria classification in a span of 4 years, like that of treated diabetes; this drift into microalbuminuria was observed in individuals with high-normal albumin levels (5-7).

Microalbuminuria is attributed to impaired permeability of the glomerulus, basic filtration unit of nephron. Glomerular filtration barrier is made up of three layers that constitutes of endothelial cells, basement membrane (lamina) and podocytes with their foot processes along the urinary side. Podocytes are connected to each other via actin-based foot processes that forms an intercellular junction known as slit diaphragm made up of nephrin, neph1 and Podocin protein. Nephrin, a 180-KD trans-membrane protein forms the main component of slit diaphragm. Nephrin is arranged in a repetitive, precise pattern that form pores that allows filtration of blood and prevents albumin and macromolecules from filtration. The cytoplasmic portion of nephrin protein regulate cytoskeletal organization and shape of the foot processes and any disruption of podocyte structure results in characteristic changes like effacement of foot processes, microvillous transformation, and occasional detachment from the glomerular basement membrane. Podocyte is the primary target of injury in Minimal change disease, focal segmental glomerulosclerosis, membranous glomerulopathy, hypertension, diabetes mellitus and lupus nephritis (8-11). In our hospital, we

encountered several newly diagnosed hypertensive patients with mild proteinuria. We wanted to evaluate whether a biomarker such as nephryn can be used for detection of early glomerular injury in treatment naïve hypertensives.

## MATERIALS AND METHODS

A prospective, cross-sectional study was conducted jointly by Department of Biochemistry and General Medicine in a tertiary care multispecialty hospital. Forty newly diagnosed hypertensive subjects were selected from General Medicine outpatient and in-patient departments. All pregnant women, known case of renal disorder, drug history that could cause hyperkalemia were excluded. Forty normotensive subjects were selected as controls. Study population belonged to age group of 20 to 80 years and included both genders.

After obtaining consent, mid-stream urine sample was collected and centrifuged at 3500 rpm to evaluate for microalbumin, creatinine and nephryn. Urine microalbumin was analyzed using Particle Enhanced turbidimetric inhibition immunoassay using dedicated reagents for fully automated Siemens Xpand automated analyzer with an assay measurement range (AMR) of 1.3 to 100 mg/L and analytical sensitivity of 1.3 mg/L, between-day and within-day imprecision below 10%. Urine creatinine was analyzed using Modified Jaffe's reaction (IFCC- IDMS traceable method) by photometric method using dedicated reagents for Siemens Xpand automated analyzer with an AMR of 5 to 400 mg/dL and analytical sensitivity of 5 mg/dL, within-day and between-day imprecision < 5 %. Urine microalbumin to creatinine ratio (ACR) was expressed as mg of albumin per gram of creatinine. Nephryn was analyzed utilizing human NPHN (nephryn) ELISA Kit, cat. No. E-EL-H1901 Elabscience Biotech Co. Ltd., Wuhan, Hubei Province, China. Human NPHN antibody was utilized with analytical sensitivity of 0.1 ng/mL and calibrated across 0.16 to 10 ng/mL with intra assay and inter assay imprecision less than 10%.

Blood sample was collected to evaluate glycated hemoglobin (HbA1c) by HPLC using BioRad Variant II Turbo Hemoglobin analyzer with AMR of 3.5 to 19% and analytical sensitivity 3.5%, within-day and between-day imprecision below 5%. As a part of standard of care, subject's renal profile consisting of urea, creatinine, sodium, and potassium in serum were analyzed. Serum Urea was analyzed using Urease/GLDH method with an AMR 0-321 mg/dL, analytical sensitivity of 1 mg/dL, within-day and between-day imprecision below 5%. Serum creatinine was analysed using Modified Jaffe's reaction (IFCC-IDMS traceable method) method using dedicated reagents for Siemens Xpand automated analyzer; serum creatinine AMR was 0.15 to 20 mg/dL with an

analytical sensitivity of 0.15 mg/dL, within-day and between-day imprecision <5 %. Estimated Glomerular Filtration Rate (eGFR) was calculated using Cockcroft-Gault formula (Creatinine Clearance (CrCl) =  $K \times \{([140 - \text{age}] \times \text{weight in kg}) / (\text{Serum creatinine} \times 72)\}$ , wherein K=1 in male and K=0.85 in female. Serum sodium and potassium were analysed using Integrated multisensory technology (IMT) on Siemens Xpand automated analyser. Sodium and potassium had an analytical sensitivity of < 50 mEq/L and <1 mEq/L respectively with an AMR of 50 to 200 mEq/L and 1 to 10 mEq/L respectively. Data collected was verified for normal distribution with the help of kolmogorov-smirnov test. Parameters which passed the normality test was described as Mean  $\pm$  Standard Deviation (SD). Data with skewed distribution was described as Median and interquartile range (IQR at Q1 and Q3). Different groups were compared using ANOVA. Odd's ratio was calculated for urine nephryn. Statistical significance was defined as p value < 0.05.

## RESULTS

Subjects were divided into 4 groups. Apparently healthy, normotensive subjects were included under group 1 or controls. Hypertensive subjects were sub-grouped based on Urinary ACR (mg/g of creatinine) as group 2: normoalbuminuria (< 30 mg/g), group 3: microalbuminuria (30 to 300 mg/g) and group 4: macroalbuminuria (> 300 mg/g). The demographic data and biochemical parameters are described in Table 1. Number of diabetic patients in group 2, 3 and 4 were 44 % (n=11), 75% (n=6) and 71 % (n=5) respectively. Duration of diabetes ranged from 2-5 years. eGFR showed significant difference between group 1 and 4 (p value < 0.05) while there was no significant difference between group 1, 2 and 3. Although there was no difference in serum urea, sodium and potassium concentration between the groups, serum creatinine showed slight elevation in subjects with macroalbuminuria. Urine microalbumin and creatinine showed a skewed distribution in the hypertensive patients, and it has been described as median and IQR. Urine nephryn excretion is comparatively higher in hypertensive subjects than normotensive subjects which was found to be statistically significant (p<0.05). To evaluate for significant nephrinuria between subjects with albuminuria and normoalbuminuria, odd's ratio was calculated. Apparently healthy normotensive subjects and normoalbuminuric hypertensive subjects were grouped together while group 3 and 4 were grouped as albuminuria group. It was found to be 29.3% with 95% confidence interval (3.61 to 238.18) and p<0.05. The hypertensive group was classified as diabetic and non-diabetic hypertensives and compared with apparently healthy controls (normotensive) (Fig.1).

**Table 1:** Demographic characteristics and biochemical parameters of study participants

Parameters	Group 1 Normotensive subjects/controls	Group 2 Hypertensive with normoalbuminuria	Group 3 (Hypertensive with microalbuminuria)	Group 4 (Hypertensive with macroalbuminuria)
Number of subjects	40	25	8	7
Age <sup>†</sup> (years)	31.5 ± 9.5	43.2 ± 6.8	51.8 ± 4.7	52.5 ± 7.4
Diabetes Mellitus	Nil	11	6	5
Systolic Blood Pressure <sup>†</sup> (mm Hg)	128 ± 11	145 ± 7	155 ± 7	195 ± 21
Diastolic Blood Pressure <sup>†</sup> (mm Hg)	74 ± 7	89 ± 2	100 ± 14	115 ± 7
HbA1c <sup>†</sup> (%)	5.9 ± 0.71	6.8 ± 0.71	7.6 ± 1.13	8.4 ± 1.98
Serum urea <sup>†</sup> (mg/dL)	24.5 ± 74.85	33 ± 9.9	40 ± 2.83	40.5 ± 6.36
Serum creatinine <sup>†</sup> (mg/dL)	0.93 ± 0.13	0.83 ± 0.27	1.10 ± 0.13	1.14 ± 0.35
eGFR <sup>†</sup> (ml/min)	96.3 ± 19.9	102.85 ± 5.59	104.8 ± 22.49	78.9 ± 9.31*
Serum Sodium <sup>†</sup> (mmol/L)	138 ± 8.8	136 ± 4.6	135 ± 7.4	134 ± 8.2
Serum Potassium <sup>†</sup> (mmol/L)	4.4 ± 2.1	4.2 ± 1.8	5.0 ± 1.4	5.1 ± 0.6
Urine microalbumin (mg/L)	13.8 ± 8.68 <sup>†</sup>	12.0 (5.3, 30.2) <sup>††</sup>	98.1 (34.5, 158.1) <sup>††</sup>	116.36 (109.24, 277.43) <sup>††</sup>
Urine Creatinine (mg/dL)	86 ± 40.4 <sup>†</sup>	78.3 (44.5, 226.7) <sup>††</sup>	89.9 (66.0, 192.3) <sup>††</sup>	23.5 (18.6, 70.3) <sup>††*</sup>
Urine ACR (mg/g of creatinine)	15.98 ± 6.50 <sup>†</sup>	16.73 (11.39, 28.48) <sup>††</sup>	48.42 (37.75, 177.05) <sup>††*</sup>	495.25 (369.49, 622.48) <sup>††*</sup>
Urine Nephryn <sup>††</sup> (ng/g of creatinine)	0.07 (0.04, 0.09)	0.15 (0.12, 0.17) *	0.8 (0.46, 1.24) *	6.24 (2.98, 9.21) *

<sup>†</sup>Mean ± SD    <sup>††</sup>Median (Q1, Q3)    \* p value < 0.05, statistically significant

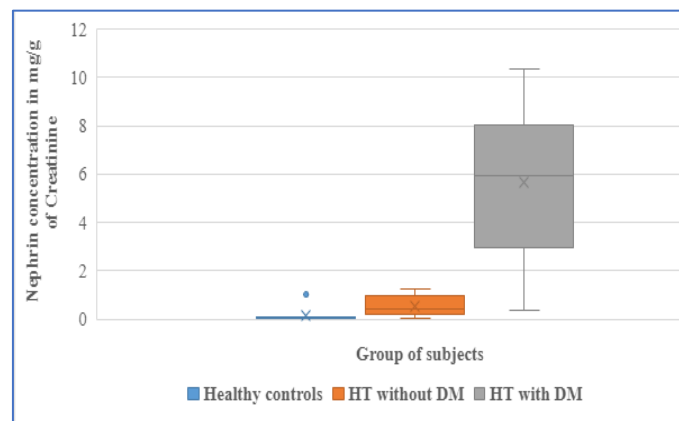


Fig.1 Urine nephryn in hypertensive and control group

## DISCUSSION

Prevalence of lifestyle diseases has been increasing with increase in life expectancy in India that is comparable to western countries. After diabetes mellitus, hypertension is the second leading cause of renal damage and dysfunction (2,3) and both account for 40 to 60% cases of chronic kidney disease (CKD) in India (12). Prevalence of hypertension in Indian adults is 17% (14.8%, rural and 21.4% urban) and diabetes is 28% in urban and 7.1% in rural population. A study in rural Karnataka, showed 3.82% prevalence of diabetes and 33.62% of hypertension and 6.3% prevalence of CKD stage 3 (13). Though a small

proportion of CKD patients reach ESRD, these patients are 10 to 100 times vulnerable for cardiovascular events. It is beneficial to identify them early and institute a preventive measure planned for cardiovascular events (14). In our study, we wanted to identify a biomarker for early glomerular injury in treatment naïve hypertensive patients to initiate measures to prevent and manage kidney dysfunction at an early stage.

Urine nephryn concentration was found to be significantly higher in hypertensive patients in comparison to apparently healthy subjects. In hypertensives, elevated systemic blood pressure might

result in elevated pressure in glomeruli giving rise to injury to podocyte due to mechanical stretching (15). Damaged podocytes result in alterations of the slit diaphragm, foot process reorganization with fusion of slits and apical displacement and if this condition persists, progressive glomerular injuries can ensue (16). It may be probably because of low Podocyte cell turnover with limited replication potential and the outcome of such injuries is detrimental (8). Nephrynuria was seen in normoalbuminuric hypertensive subjects and was statistically significant in comparison to controls. The odds of developing nephrynuria are 29.3% times higher and 95% confidence interval was wide indicative of inadequate sample size. Normoalbuminuric subjects presenting with increased levels of urine nephryn could be indicative of status that precedes albuminuria and loss of glomerular function. Tubular theory proposes impaired tubular uptake of intact albumin leads to albuminuria rather than increased leakiness in glomerular filtration barrier (17). Urine nephryn concentration is lower in hypertensive subjects without diabetes mellitus when compared to combination of diabetes and hypertension in subjects. Our study findings are comparable to reports by Jim *et al.*, (18) who showed that nephrynuria was seen in 54% of patients with normoalbuminuria and 100% of diabetic patients with microalbuminuria and macroalbuminuria. Nephrynuria correlated with systolic blood pressure and it was reported that nephryn is a reliable early biomarker of diabetic nephropathy (18).

A cut-off value for urine nephryn was considered as 0.09 mg/g of creatinine as seen in healthy control. The cut-off found in several studies were slightly variable though comparable (19-21) and this could be attributed to different ELISA kits used by researchers. Large population study is required to establish cut-off for urine nephryn concentration.

Urine ACR was significantly higher in group 3 and 4 (48.42 and 495.25 mg/g of creatinine respectively) and this finding is similar to Kondapi *et al.*, (20). There is proportionate rise in serum creatinine (not statistically significant) and associated decrease in eGFR in group 4. It could be due to small sample size and requires to be evaluated in larger study population.

Serum urea did not show significant difference across groups. Dietary protein intake is directly proportional to urea production and since we did not document the dietary recall or fluid intake for subjects and it is one of the limitations in our study.

Serum sodium and potassium were not significantly different between groups although a small proportion of subjects in group 4 showed sodium levels lower than 125 mEq/L and potassium beyond 5.5 mEq/L. Several studies have shown low serum sodium and high potassium levels associated with hypertension (21, 22).

Well controlled and monitored blood pressure can prevent decrease in glomerular filtration rate in diabetic and non-diabetic patients (23,24). However, some of these studies indicate in addition to blood pressure, proteinuria should be under tight control for prevention from renal and cardiovascular protection (23-25).

Our study had several limitations: a) sample size was small in sub groups for treatment naïve hypertensive subjects. b) Dietary and fluid intake recall was not documented for the subjects. c) this was cross-sectional and involved only a single glomerular protein nephryn. Strength of our study is that we have showed there is urine nephryn loss in hypertensives earlier than albuminuria. There are very few studies on nephryn as a biomarker in glomerular injury amongst treatment naïve hypertensives in Indian population.

## CONCLUSION

In conclusion, our study demonstrates that urine nephryn can be used as a biomarker of early glomerular injury in hypertensive subjects. Larger cohorts maybe required for further validation of this outcome and also to have standardised cut-off and unit of expression for urinary nephryn.

## CONFLICT OF INTEREST

None

## REFERENCES

1. Wachtell, K., Palmieri, V., Olsen, M.H., Bella, J.N., Aalto, T., Dahlof, B., *et al.*, Urine albumin/creatinine ratio and echocardiographic left ventricular structure and function in hypertensive patients with electrocardiographic left ventricular hypertrophy: The LIFE study. Losartan Intervention for Endpoint Re-duction. Am Heart J 2002; 43: 319-326.
2. Danziger, J. Importance of low-grade albuminuria. Mayo Clin Proc 2008; 83: 806-812.
3. Wang, T.J., Evans, J.C., Meigs, J.B., Rifai, N., Fox, C.S., D'Agostino, R.B., *et al.*, Low-grade albuminuria and the risks of hypertension and blood pressure progression. Circulation 2005; 111: 1370-1376.
4. Hillege, H.L., Janssen, W.M., Bak, A.A., Diercks, G.F., Grobbee, D.E., Crijs, H.J., *et al.*, PREVEND Study Group: Microalbuminuria is common, also in a nondiabetic, nonhypertensive population, and an independent indicator of cardiovascular risk factors and cardiovascular morbidity. J Intern Med 2001; 249: 519-526.
5. Brantsma, A.H., Attobari, J., Bakker, S.J., de Zeeuw, D., de Jong, P.E., Gansevoort, R.T.: What causes progression and regression of urinary albumin excretion in the general population? [Abstract]. J Am Soc Nephrol 2005; 16: 324A.
6. Adler, A.I., Stevens, R.J., Manley, S.E., Bilous, R.W., Cull, C.A., Holman, R.R.: Development and progression of nephropathy in type 2 diabetes: The United Kingdom Prospective Diabetes Study (UKPDS 64). Kidney Int 2003; 63: 225-232.
7. Ruggerenti, P., Fassi, A., Ilieva, A.P., Bruno, S., Iliev, I.P., Brusegan, V., *et al.*, Bergamo Nephrologic Diabetes Complications Trial (BENEDICT) Investigators; Preventing microalbuminuria in type 2 diabetes. N Engl J Med 2004; 351:1941-1951.

8. Kerjaschki, D. The pathogenesis of membranous glomerulonephritis: From morphology to molecules. *Virchows Arch (B)* 1990; 58: 253-271.
9. Rennke, H.G., Klein, P.S. Pathogenesis, and significance of nonprimary focal and segmental glomerulosclerosis. *Am J Kidney Dis* 1989; 13: 443-456.
10. Olson, J.L., Heptinstall, R.H. Nonimmunologic mechanisms of glomerular injury. *Lab Invest* 1988; 59: 564 -576.
11. Andrews, P. Morphological alterations of the glomerular (visceral) epithelium in response to pathological and experimental situations. *J Electron Microsc Tech* 1988; 9: 115-144.
12. Rajapurkar, M.M., John, G.T., Kirpalani, A.L., Abraham, G., Agarwal, S.K., Almeida, A.F., *et al.*, What do we know about chronic kidney disease in India: first report of the Indian CKD registry. *BMC Nephrol.* 2012; 6: 13:10.
13. Varma, P.P. Prevalence of chronic kidney disease in India - Where are we heading? *Indian J Nephrol.* 2015; 25 (3)133-135
14. Tonelli, M., Wiebe, N., Culleton, B., House, A., Rabbat, C., Fok, M., *et al.*, chronic kidney disease and mortality risk: a systematic review. *J Am Soc Nephrol.* 2006; 17 (7):2034-2047.
15. Kato, T., Mizuguchi, N., Ito, A. Characteristics of podocyte injury in malignant hypertensive nephropathy of rats (MSHRSP/Kpo strain) *Biomed Res.* 2015; 36: 313-321.
16. Kandasamy, Y., Smith, R., Lumbers, R.E., and Rudd, D. Nephlin - a biomarker of early glomerular injury. *Biomarker Res.* 2014; 2: 21
17. Russo, L.M., Sandoval, R.M., Campos, S.B., Molitoris, B.A., Comper, W.D., Brown, D. Impaired tubular uptake explains albuminuria in early diabetic nephropathy. *J AM Soc. Nephrol.* 2009; 20: 489-494.
18. Jim, B., Ghanta, M., Qipo, A., Fan, Y., Chuang, P.Y., Cohen, H.W., *et al.*, Dysregulated nephlin in diabetic nephropathy of type 2 diabetes: A cross sectional study. *PLoS One* 2012; 7:5:e36041.
19. Petricia, L., Ursoniu, S., Gadalean, F., Vlad, A., Gluhovschi, Dumitrascu, V., *et al.*, Urinary podocyte-associated mRNA levels correlate with proximal tubule dysfunction in early diabetic nephropathy of type diabetes mellitus. *Diabetol Metab Syndr.* 2017; 9: 31.
20. Kondapi, K., Kumar, N. L., Moorthy, S., Silambanan, S. A study of association of urinary nephlin with albuminuria in patients with diabetic nephropathy. *Indian J Nephrol* Available from: <https://www.indianjephrol.org/preprintarticle.asp?id=309896>
21. Kondapi, K., Silambanan, S. and Moorthy, S. Diagnostic sensitivity of biomarkers in assessing the occurrence and progression of diabetic nephropathy. *Eur J of Molecular and Clinical Medicine.* 2021; 8 (3): 1554-1571.
22. Zhang, Z., Cogswell, M.E., Gillespie, C., Fang, J., Loustalot, F., Dai, S., *et al.*, Association between usual sodium and potassium intake and blood pressure and hypertension among U.S. adults: NHANES 2005–2010. *PLoS One* 2013; 8: e75289.
23. Mane, A.S. Correlation between serum sodium and potassium levels and risk of developing hypertension. *International Journal of Contemporary Medical Research* 2018; 5 (11): K5-K9.
24. Jafar, T.H., Stark, P.C., Schmid, C.H., Landa, M., Maschio, G., de Jong, P.E., *et al.*, Progression of chronic kidney disease: the role of blood pressure control, proteinuria, and angiotensin-converting enzyme inhibition: a patient-level meta-analysis. *Ann Intern Med* 2003; 139: 244-252.
25. Maione, A., Annemans, L., Strippoli, G. Proteinuria and clinical outcomes in hypertensive patients, *American Journal of Hypertension* 2009; 22 (11): 1137-1147.